JOURNAL OF BACTERIOLOGY, Feb. 1994, p. 569–579
0021-9193/94/$04.00+0

Vol. 176, No. 3

A Low-Ca^{2+} Response (LCR) Secretion (*ysc*) Locus Lies within the *lcrB* Region of the LCR Plasmid in *Yersinia pestis*

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Received 11 August 1993/Accepted 23 November 1993

The causative agent of plague, *Yersinia pestis*, contains a 75-kb plasmid, pCD1, which carries a virulence-related stimulon called the low-Ca^{2+} response stimulon (LCRS). LCRS operons are regulated by the environmental signals of temperature and Ca^{2+}. This study characterized a portion of the *lcrB* region of pCD1, known to contain at least one gene necessary for the regulation of LCRS operons by Ca^{2+}. The sequence of a 2-kb region revealed three open reading frames, designated *yscQ*, *yscR*, and *yscS*, predicted to encode acidic proteins of 34.4, 24.4, and 8.5 kDa. All three proteins were homologous to proteins involved in flagellar function or virulence. An antipeptide antibody specific for YscR was used to localize YscR to the inner membrane of *Y. pestis*. Analysis of *yscR-phoA* fusions supported a model for *yscR* which predicts four transmembrane regions and a large, central hydrophilic domain. In-frame deletion mutations of *yscQ* and *yscR* were constructed and moved into *Y. pestis*. Both mutants failed to show the restriction of growth that normally accompanies maximal LCRS induction. Unlike the parent *Y. pestis*, the *yscR* mutant did not respond to the absence of Ca^{2+} by increasing the net transcription or translation of the LCRS-encoded V antigen, YopM, or LcrG. The *yscR* mutant also was defective for secretion of V antigen, YopM, and LcrG. These findings implicate a dual role for YscR in regulation of LCRS operons and secretion of LCRS proteins and add to the developing picture of how secretion of virulence proteins may be coupled to transcriptional regulation in yersiniae.

Three species of the genus *Yersinia*, *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, are pathogenic to humans. *Y. pestis* causes bubonic plague (13), while *Y. pseudotuberculosis* and *Y. enterocolitica* cause invasive gastrointestinal disease (14). Although *Y. pestis* causes a more systemic infection, it shares certain virulence features with the enteropathogenic yersiniae. One common feature is a virulence-associated regulatory response called the low-Ca^{2+} response (LCR), which is encoded by highly related ca. 75-kb plasmids (78). The LCR brings about the coordinate regulation of plasmid-encoded virulence genes by environmental temperature and Ca^{2+} or nucleotides (11, 35, 78). These genes, which belong to the LCR stimulon (LCRS), encode secreted factors such as V antigen (12, 79) and a set of about 10 virulence proteins called Yops (9, 58, 76, 77, 79). Proteins regulating the expression and secretion of V antigen and Yops are encoded within a ca. 25-kb region of the LCR plasmid termed the Ca^{2+} dependence region (9, 13, 24, 57, 78, 83).

One manifestation of the LCR is the requirement for millimolar calcium or 10 to 20 mM nucleotides, such as ATP, for growth above 34°C (28, 84, 85). Although growth occurs independently of Ca^{2+} or ATP at 26°C, a shift in incubation temperature to 37°C in the absence of Ca^{2+} or ATP results in a cessation of growth within a few generations (28, 84). This phenomenon is termed growth restriction. Paradoxically, restriction is concurrent with maximal induction of LCRS operons and secretion of LCRS-encoded proteins.

V antigen is a soluble protein encoded by the *lcrV* gene of the *lcrGVH-yopBD* operon (5, 52, 59). Although a specific antihost function has not been assigned to V antigen, it is a protective antigen and is believed to be required for full virulence (10, 36, 73). Yops are encoded by multiple operons (15, 79), and functions that have been identified for them include cytotoxicity (66, 67), inhibition of phagocytosis (65, 66), and sequestration of thrombin (38, 64). V antigen and Yops lack cleavable N-terminal signal sequences and are secreted by an LCRS-encoded mechanism (45). N-terminal sequences have been implicated in directing transport from the cell (43).

Previously, two loci within the Ca^{2+} dependence region have been shown to affect both LCRS operon transcription and secretion. LcrD of the *lcrDR* operon is necessary for maximal induction of LCRS operons and secretion of LCRS-encoded virulence proteins (24, 54, 55). LcrD has homology to several proteins associated with flagellar biogenesis, invasion of epithelial cells, and plant pathogenesis in taxonomically divergent bacteria (54, 78). The LcrD homolog is thought to be a component of a novel secretion system involved in each of these diverse processes.

The 13-cistron *ysc* locus is also necessary for the secretion of the V antigen and Yops (27, 44). Polar insertion mutants of this region are defective in LCR-specific secretion and show altered regulation of LCRS operon transcription (24, 27, 44). However, the dominant effects on regulation are the opposite between *Y. pestis* and the enteropathogenic yersiniae, with constitutive downregulation being seen in the former and constitutive induction being seen in the latter (24, 27, 44). This could arise from different extents of polarity in the respective *ysc* loci, and sorting out these differences will require analysis of nonpolar mutants in the various *ysc* genes.

Early studies involving the mutagenesis of the *Y. pestis* LCR plasmid with bacteriophage MudI1 (Ap^r *lac*) identified a third locus, *lcrB*, required for expression of the LCR (24). This region had not been characterized further. In this study, we investigated a portion of the *lcrB* region to determine its function in the LCR. We provide evidence that at least one protein encoded by this region is required, like LcrD, for induction of LCRS operons and secretion of V antigen and Yops. In addition, we report that several proteins encoded by

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Properties	Source or Reference
E. coli K-12		
XL1-Blue	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96* (Nal^r) *relA1* (*lac*) [F$^+$ *proAB*$^+$ *lacI*^{q}*Z* ΔM15::Tn*10*(Tc^r)]	Stratagene
SY327 λ*pir*	Δ(*lac pro*) *argE*(Am) *rif nalA recA56* λ*pir*	47
DH5αF′ λ*pir*	F′/*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ)(*lacZYA-argF*)U169 *deoR* (ϕ80d*lac*Δ[*lacZ*]M15) λ*pir*	Gibco-BRL
CC118	Δ(*ara-leu*)*7697 araD139* Δ*lacX74 galE galK* Δ*phoA20 thi rpsE rpoB argE*(Am) *recA1*	42
Y. pestis		
KIM5-3001	Sm^r; pCD1[a] (Lcr$^+$), pPCP1, pMT1	39
KIM5-3001.7	Sm^r; pCD1 *yscQ* (YscQΔ4-68[b]), pPCP1, pMT1	This study
KIM5-3001.8	Sm^r; pCD1 *yscR* (YscRΔ73-106[b]), pPCP1, pMT1	This study
Plasmids		
pSK$^-$	Ap^r; cloning vector	Stratagene
pUK4134	Ap^r; suicide vector *ori*R6K *ori*T *cos rpsL*	72
pBGCD1	Ap^r; *Bgl*II A fragment of pCD1 ligated into *Bgl*II-cut pBGL2	Robert D. Perry
Derivatives in pSK$^-$		
pYP-F1	Ap^r; *Bam*HI F fragment cloned from pBGCD1; *ysc* oriented with the T7 promoter; contains *yscQRS*, with frameshift mutation in *yscR*	This study
pYP-K1	Ap^r; *Hin*dIII K fragment cloned from pYP-F1; *ysc* oriented with the T7 promoter; contains *yscQRS′* with frameshift mutation in *yscR*	This study
pYPK	Ap^r; *Sfu*I-*Afl*II internal fragment of *yscR* in pYP-K1 replaced with *Sfu*I-*Afl*II fragment from pYP-D4; *ysc* oriented with T7 promoter; contains *yscQRS′*	This study
pYPK.R	Ap^r; *Hin*dIII K fragment of pYPK with *ysc* oriented with *lac* promoter and against the T7 promoter; contains *yscQRS′*	This study
pYP-F(Q:Δ4-68)	Ap^r; *Hpa*I deletion of pYP-F1; *Hpa*I cut and religated; contains *yscQ* (YscQΔ4-68), *yscR* with a frameshift mutation, and *yscS*	This study
pYPK.R(Q:Δ4-68)	Ap^r; *Hpa*I deletion within pYPK.R made by cutting with *Hpa*I and religating; *yscQ* (YscQΔ4-68) *yscRS′*	This study
pYPK (R:Δ73-106)	Ap^r; *Nru*I-*Afl*II digestion of pYPK; ends filled in with Klenow fragment and religated; *yscR* (YscRΔ73-106)	This study
pYP-D4	Ap^r; PCR product from pCD1 containing nucleotides 873–1644 (Fig. 2); kinase treated and ligated into *Eco*RV-cut and phosphatase-treated pSK$^-$	This study
pYEC20.1	Ap^r; PCR product from LCR plasmid of *Y. enterocolitica* WA (O:8) containing nucleotides corresponding to 1129–1378 (Fig. 2); kinase treated and ligated into *Eco*RV-cut and phosphatase-treated pSK$^-$	This study
pYTB14.1	Ap^r; PCR product from LCR plasmid of *Y. pseudotuberculosis* PB1/+ (serotype I) containing nucleotides corresponding to 1129–1378 (Fig. 2); kinase treated and ligated into *Eco*RV-cut and phosphatase-treated pSK$^-$	This study
Derivatives in pUK4134		
pUK4134.5	Ap^r; *Cla*I-*Sfu*I digestion of pYP-F(Q:Δ4-68) followed by fill-in with Klenow fragment and ligation into *Eco*RV-cut and phosphatse-treated pUK4134	This study
pUK4134.6	Ap^r; *Hin*dIII digestion of pYPK(R:Δ73-106) followed by fill-in with Klenow fragment and ligation into *Eco*RV-cut and phosphatase-treated pUK4134	This study

[a] Native *Y. pestis* KIM virulence plasmids are the LCR plasmid pCD1 (24), pPCP1 (60, 75), and pMT1 (60).
[b] Numbers represent amino acids deleted from the protein product.

this locus are homologous to families of proteins with members variously implicated in flagellar function, secretion of virulence proteins, or expression of virulence.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions. Bacterial strains and their relevant properties are listed in Table 1. *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) was used as a general host for cloned DNA and for analysis of protein products with the T7 promoter/polymerase expression system. *E. coli* SY327 λ*pir* (47) and DH5α λ*pir* (Gibco-BRL, Gaithersburg, Md.) were used as hosts for derivatives of the suicide vector pUK4134. λ::Tn*phoA* mutagenesis was carried out in *E. coli* CC118 (42). The resulting clones were also maintained in this strain. All *Y. pestis* strains were *pgm* and therefore avirulent by peripheral routes but highly virulent from an intravenous route (80). The M13 bacteriophage derivative mGP1-2, which encodes the T7 RNA polymerase, was used for expression of cloned DNA under the influence of a vector T7 promoter.

E. coli strains typically were grown in L broth (17) or on L agar. L broth supplemented with 1% (wt/vol) maltose and 10 mM $MgSO_4$ was used for growth of *E. coli* CC118 before infection with λ::Tn*phoA*. M9 minimal medium (41) was used for T7 promoter/polymerase expression experiments. *Y. pestis* was commonly grown in heart infusion broth or on tryptose blood agar base (Difco Laboratories, Detroit, Mich.). The defined medium TMH (76) was used during analysis of growth characteristics and for cultivation of bacteria subsequently analyzed for protein or RNA expression. The antibiotics ampicillin, tetracycline, kanamycin, and streptomycin were used where appropriate at concentrations of 100, 25, 50, and 50 μg/ml, respectively.

DNA methods and plasmid constructions. Cloning methods, including the use of restriction endonucleases, were performed essentially as described by Maniatis et al. (41). Plasmid DNA

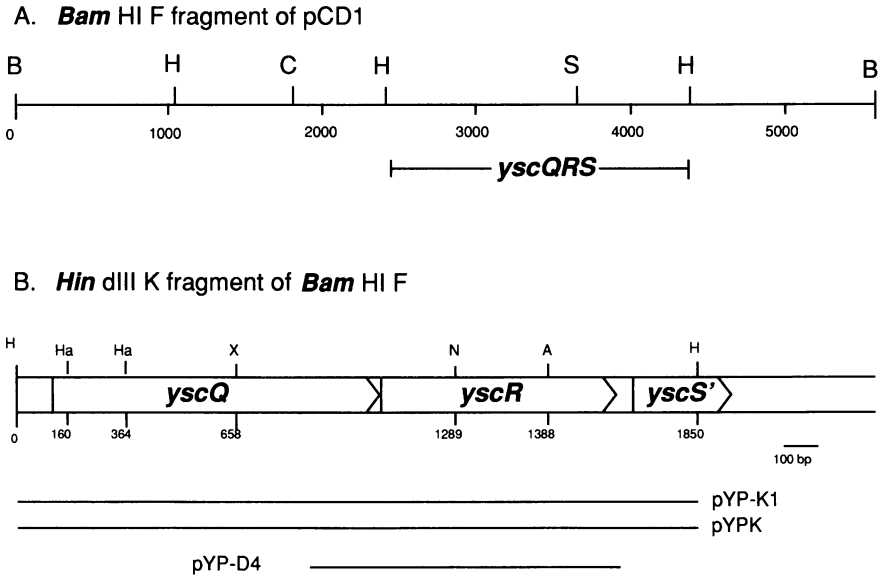

FIG. 1. Physical and genetic maps of a portion of the *lcrB* region of *Y. pestis* LCR plasmid pCD1. (A) The *Bam*HI F fragment of pCD1 contained in pYPF1. The locations of *yscQ*, *yscR*, and *yscS* are noted. Numbers indicate sizes in nucleotides. (B) The *yscQRS'*-containing pCD1 *Hin*dIII K fragment derived from *Bam*HI-F. The coding regions for *yscQ* and *yscR* and part of *yscS* as well as selected restriction sites, are shown. Numbers correspond to nucleotide numbers in Fig. 2. (C) Regions included in selected clones. Abbreviations for restriction endonucleases: A, *Afl*II; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; Ha, *Hpa*I; N, *Nru*I; S, *Sfu*I.

was isolated by an alkaline lysis procedure (6), by the method of Kado and Liu (33), or with Qiagen columns (Qiagen, Inc., Studio City, Calif.). Certain DNA restriction fragments were purified from agarose after electrophoresis with the Qiaex gel extraction kit (Qiagen). Both *E. coli* and *Y. pestis* strains were transformed via electroporation as previously described (53). PCR (49) was performed by using 18-nucleotide primers and 30 cycles of amplification. Denaturing, annealing, and extending conditions were 94°C for 1 min, 42°C for 2 min, and 72°C for 2 min, respectively.

Plasmid pYP-F1 was derived from pBGCD1, a cosmid clone, kindly provided by Robert D. Perry, University of Kentucky, containing the 18-kb *Bgl*II A restriction fragment from pCD1. Unless otherwise noted, restriction fragments were cloned into the cloning vector pBluescript SKII$^-$ (pSK$^-$). pYP-F1 contained the 5.2-kb *Bam*HI fragment within pBGCD1. This fragment corresponds to the *Bam*HI F fragment of the *Y. pestis* LCR plasmid pCD1 (Fig. 1). Several further subclones were constructed from pYP-F1. pYP-F(Q:Δ4-68) was made by deletion of an internal 204-bp *Hpa*I fragment from *yscQ* (Fig. 1B). pYP-K1 contained a 1.8-kb *Hin*dIII fragment from pYP-F1 (corresponding to *Hin*dIII-K of pCD1; Fig. 1C).

During the course of this study, we discovered that either pYP-F1 or the original pCD1 subclone, pBGCD1, had suffered a point mutation. The result was the addition of a cytosine to a stretch that normally contained seven, shifting the reading frame. The correct number of cytosines was established by sequencing from pYP-D4, which was derived directly from pCD1 by PCR (Fig. 1C). Further verification was made by using pYEC20.1 and pYTB14.1, which contained PCR products derived from the LCR plasmids of *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively. The PCR fragments were generated by using primers based on sequence in the *lcrB* (*ysc*) region of pCD1 (Table 1). The mechanism giving rise to the frameshift mutation was not pursued.

We corrected the mutated region in affected subclones by replacing a 347-bp *Sfu*I-*Afl*II fragment, which contained the frameshift mutation, with the *Sfu*I-*Afl*II fragment from pYP-D4, which possessed the correct number of cytosines. The resulting clone was called pYPK (Fig. 1C). It was partially sequenced to confirm that the correct number of cytosines was present. pYPK.R was made by *Hin*dIII digestion and religation of pYPK. It contained the *Hin*dIII fragment in the opposite orientation in comparison with pYPK. These two clones were used in subsequent analysis of the *lcrB* region (later given a *ysc* designation since it is a second *ysc* operon). Plasmid pYPK.R(Q:Δ4-68) was derived by deletion of the 204-bp *Hpa*I fragment from *yscQ* in pYPK.R (Fig. 1B).

Two plasmids were constructed by using the suicide vector pUK4134. pUK4134.5 contained a Klenow fragment-treated *Cla*I-*Sfu*I fragment of pYPF(Q:Δ4-68), beginning upstream of *yscQ*, including the 204-bp in-frame *Hpa*I deletion in *yscQ*, and ending near the end of *yscQ* (Fig. 1A shows these restriction sites). pUK4134.6 carried the filled-in and blunt-end-cloned *Hin*dIII fragment from pYPK(R:Δ73-106), containing the in-frame 99-bp *Nru*I-*Afl*II deletion in *yscR* (Fig. 1B). pUK4134.5 and pUK4134.6 were used to construct the mutant strains *Y. pestis* KIM5-3001.7 and KIM5-3001.8, respectively, by allelic exchange (72).

DNA sequence analysis. Double-stranded DNA was sequenced by the method of Sanger et al. (69), using synthetic nucleotide primers, [^{35}S]dATP (ICN, Costa Mesa, Calif.), and the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio). Computer analysis of the resulting sequence was done with PCGene programs (IntelliGenetics, Inc., Mountain View, Calif.). Transmembrane regions of protein sequences were predicted by using the programs GARNIER (23) and HELIXMEM (19). Amino acid sequences were searched by the BLASTX program (IntelliGenetics) for homology to existing proteins in the SwissProt data base.

Protein expression and topology analysis. We tested expres-

sion of predicted coding sequences by using a bacteriophage T7 promoter/polymerase system as previously described (54). Both boiled and nonboiled samples of labeled, clone-specific and vector-specific proteins from solubilized cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent autoradiography. Mutagenesis of plasmid pYPK with Tn*phoA* was performed essentially as described by Plano et al. (54). Cultures of CC118 transformed with pYPK were infected at a multiplicity of 2 with λ::Tn*phoA* and then incubated at 37°C for 3 h. Clones containing single copies of Tn*phoA* were screened, and fusion junctions were determined by sequencing from double-stranded DNA as described previously (54). Units of alkaline phosphatase (AP) activity were derived on the basis of hydrolysis of *p*-nitrophenyl phosphate as described by Plano et al. (54).

Antibody preparation. An antipeptide antibody was prepared against residues 27 to 38 of *yscR* (H_2N-CPKQYA DRLESDS-COOH), predicted by the ANTIGEN program (PCGene) to be antigenic. Coupling to the carrier bovine serum albumin was done with 0.20% (vol/vol) glutaraldehyde (2 h, ambient temperature). The mixture was dialyzed against phosphate-buffered saline (135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.2]) to remove glutaraldehyde, and peptide-carrier conjugates were purified via gel filtration chromatography. Antibody was raised in female New Zealand White rabbits and was harvested and purified as described by Plano et al. (54). Anti-YscR antibody was used at a dilution of 1:500 for immunoblot analysis.

Immunoblot analysis. Samples were prepared from *Y. pestis* strains grown in TMH. Medium (TMH with and without 2.5 mM Ca^{2+}) was inoculated to an A_{620} of 0.10 from a culture that had been growing exponentially at 26°C for about seven generations. Cultures were started at 26°C and then shifted to 37°C after 2 h. Bacteria (18.65 OD ml) were harvested by centrifugation 5 h later. Supernatant medium was passed through 0.22-μm-pore-size low-protein-binding filters (Millipore Corp., Bedford, Mass.), and secreted proteins were precipitated with 5% (vol/vol) trichloroacetic acid (1 h, on ice) and collected by centrifugation at 15,200 × *g* for 15 min. Proteins were solubilized in electrophoresis sample buffer (35, 54). Bacteria were suspended in 100 mM Tris-HCl (pH 7.4)–1 mM EDTA and lysed by a single passage through a French pressure cell at 20,000 lb/in^2. Unlysed cells were removed by centrifugation at 8,800 × *g* for 5 min. Membranes were pelleted from lysates by a 20-min ultracentrifugation at 263,800 × *g* in a TLA 100.3 rotor (Beckman, Inc., Palo Alto, Calif.). Half of the supernatant was removed and saved as the soluble fraction. Membranes were suspended in 100 mM Tris-HCl (pH 7.4)–1 mM EDTA. Inner and outer membranes were prepared in separate experiments as previously described (77). Volumes of cellular fractions corresponding to equal numbers of bacteria were mixed 1:1 (vol/vol) with 2× electrophoresis sample buffer and analyzed by SDS-PAGE.

Most samples were subjected to denaturing SDS-PAGE (12% [wt/vol] acrylamide) and transferred to Immobilon-P (Millipore) as previously described (54). Those samples to be analyzed with anti-LcrG antibody were electrophoresed on 15% (wt/vol) acrylamide gels and transferred to Immobilon-P in carbonate transfer buffer (10 mM $NaHCO_3$–3 mM Na_2CO_3 [pH 9.9] in 20% [vol/vol] methanol) (18) for 4 h at 40 V. For all samples, membranes were blocked for 2 h with 20 mM Tris-HCl–0.85% (wt/vol) NaCl (pH 8.0) containing 5% (wt/vol) nonfat dry milk. Blocking solution was replaced by appropriate antibody diluted in blocking solution containing 0.05% (vol/vol) Tween 20 and 1% (wt/vol) nonfat dry milk. Respective proteins were visualized as previously described (54), using antipeptide antibodies specific for V antigen (74), YopM (64), and LcrG (74).

Northern (RNA) analysis. *Y. pestis* KIM5-3001 and KIM5-3001.8 were grown in TMH as stated above. Bacteria were harvested 4 h after the temperature shift to 37°C, and total RNA was isolated by the method of K. J. Reddy as described in reference 3. Five micrograms of each RNA sample was separated on a 1.5% (wt/vol) agarose gel and vacuum blotted to nitrocellulose. Nitrocellulose was probed with PCR fragments derived from within *yscR* (nucleotides 1128 to 1641 in Fig. 2) and *yopM* (nucleotides 156 to 948 in Fig. 6 [38]) that had been labeled with ^{32}P by nick translation by using a kit obtained from Promega Corp. (Madison, Wis.). Hybridization and autoradiography were carried out as described elsewhere (4). A BioImage Visage 2000 densitometer (Millipore) was used to quantitate the density in lanes of the autoradiograms.

Nucleotide sequence accession number. The nucleotide sequence for *yscQRS* (Fig. 2) has been submitted to GenBank with accession number L22495.

RESULTS

Nucleotide sequence in the *lcrB* locus of *Y. pestis* KIM. The ca. 5.2-kb *Bam*HI F fragment of the *lcrB* region of pCD1 (24) was subcloned from pBGCD1 (Fig. 1), and the 1.8-kb *Hin*dIII K fragment and a portion of the downstream *Hin*dIII H fragment were sequenced (Fig. 2). Computer analysis of the sequence revealed three complete open reading frames (ORFs) of 920, 650, and 230 nucleotides, predicted to encode proteins of 34,418, 24,427, and 8,531 Da, respectively. These putative genes were designated *yscQ*, *yscR*, and *yscS* for their putative roles in Yop secretion (see below). All three *ysc* ORFs were preceded by potential ribosome binding sites that were identical to, or shared four of five residues of, the *E. coli* GGAGG consensus sequence (71). The translation stop codon of an incomplete ORF overlapped the predicted translation start codon of *yscQ*. Likewise, the translational stop and start codons of *yscQ* and *yscR* overlapped. The stop codon of *yscR* and the start of *yscS* were separated by only one nucleotide. This information and the absence of sequences that resembled prokaryotic promoter sequences suggested that *yscQRS* lies within a larger, polycistronic operon.

YscQ, YscR, and YscS were all predicted to be acidic proteins with pI values of 4.91, 4.61, and 6.03, respectively. None of the deduced proteins contained typical N-terminal signal sequences. YscQ was predicted to be a soluble or peripheral protein, while YscR and YscS were predicted to be integral membrane proteins, with four and two membrane-spanning regions, respectively.

Expression in *E. coli*. Products of the cloned *yscQ* and *yscR* genes were expressed from plasmid pYPK (Fig. 1) in *E. coli* by using a bacteriophage T7 promoter/polymerase system (Fig. 3). The subclone pYPK contained the coding sequences for YscQ and YscR. Plasmid pYPK(R:Δ73-106) was a derivative of pYPK which had an in-frame internal deletion of 99 nucleotides in *yscR*. The pSK$^-$ vector was provided as a control. Plasmid pYPK expressed a unique ca. 20,000-Da protein product which roughly corresponded in size to YscR (predicted molecular size of 24,427 Da) (Fig. 3, lanes 3 and 4). Plasmid pYPK (R:Δ73-106) did not express the 20-kDa protein or any stable deletion products (lanes 5 and 6), confirming that the 20-kDa band was the product of *yscR*. The product of the *yscQ* gene may also have been expressed; however, it may have comigrated with a strong band that appeared in the pSK$^-$

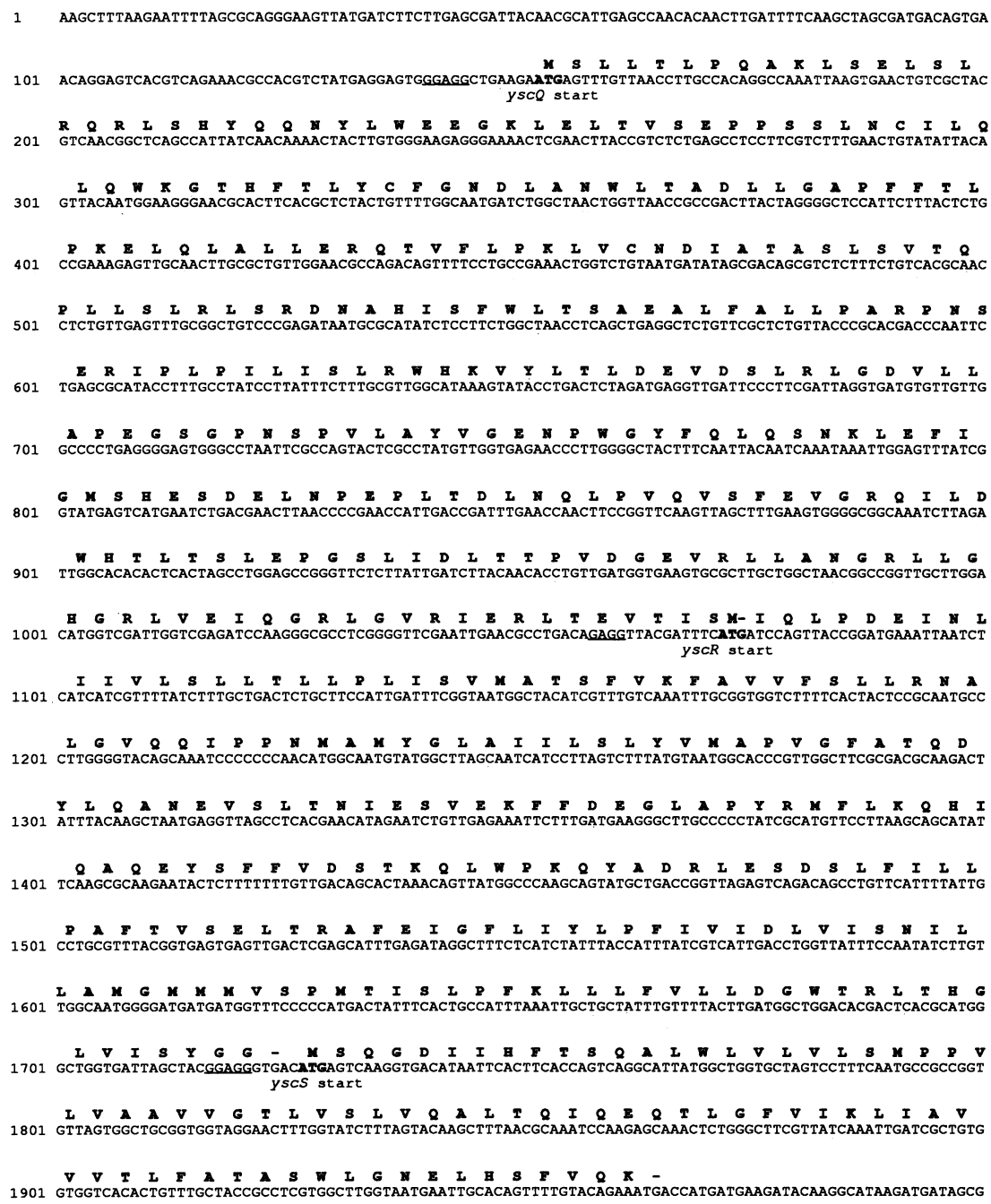

FIG. 2. Nucleotide and predicted protein sequences of a portion of the *lcrB* region. Nucleotide and translated amino acid sequences for *yscQ*, *yscR*, and *yscS* are shown. Amino acid residues are in boldface; potential ribosome binding sites are underlined.

control. The complete coding sequence for YscS was not present on pYPK.

Localization and topology of YscR. We used Western blot (immunoblot) analysis with an antipeptide antibody directed against amino acids 127 to 138 of YscR to localize YscR in *Y. pestis* (Fig. 4). Total and separated membranes from the parent *Y. pestis* KIM5-3001 and the YscR$^-$ mutant *Y. pestis* KIM5-3001.8 were analyzed for the presence of YscR. Soluble and supernatant fractions were also analyzed (data not shown). A band corresponding to YscR was observed in *Y. pestis* KIM5-3001 grown at 37°C in the absence of Ca^{2+} (Fig. 4, lanes 2, 6, and 8). Significantly, little YscR was seen when the yersiniae had been grown in the presence of Ca^{2+} (lanes 1, 5, and 7). YscR was absent in the mutant *Y. pestis* KIM5-3001.8 grown both in the presence and in the absence of Ca^{2+} (lanes 3 and 4). The analysis of inner (lanes 5 and 6) and outer (lanes 7 and 8) membrane preparations localized YscR to the inner membrane.

AP activities of YscR-PhoA translational fusions were used to provide information on the topological arrangement of YscR in the membrane of *Y. pestis* KIM5-3001. Six unique clones that had in-frame Tn*phoA* insertions in *yscR* were

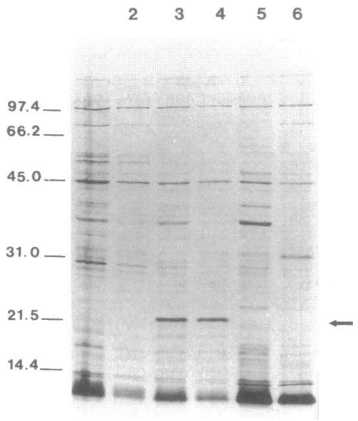

FIG. 3. SDS-PAGE protein profiles of genes expressed under the influence of a T7 promoter. Lanes: 1 and 2, vector pSK$^-$; 3 and 4, pYPK; 5 and 6, pYPK(R:Δ73-106). Samples in lanes 1, 3, and 5 were solubilized prior to electrophoresis at 100°C; samples in lanes 2, 4, and 6 were solubilized at room temperature. A band corresponding to YscR is indicated with an arrow. Numbers to the left give the approximate masses in kilodaltons of prestained molecular weight markers (Gibco-BRL). In repetitions of this experiment, a more accurate determination of the apparent molecular mass of YscR was made with reference to ^{14}C-labeled molecular weight standards (Amersham, Arlington Heights, Ill.). In those gels, the YscR band migrated at 19.5 kDa (data not shown).

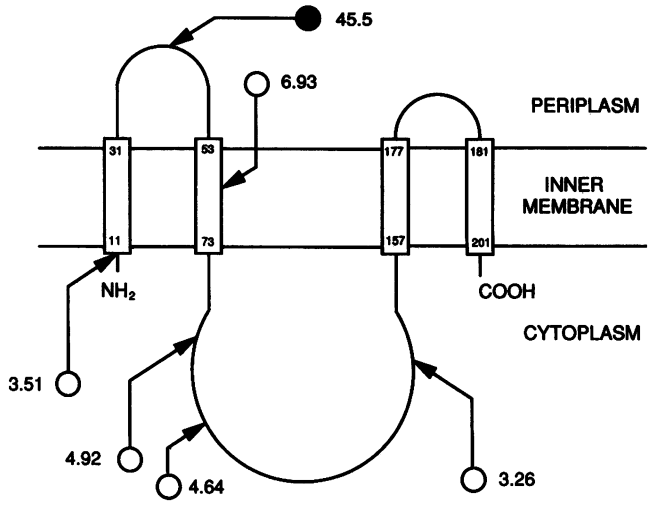

FIG. 5. Model for the configuration of YscR in the inner membrane of *Y. pestis*. Predicted transmembrane regions are depicted as rectangles; amino acid residues represented as numbers. YscR-PhoA fusions are shown with solid circles (high AP activities) or open circles (low AP activities). Insertions were after residues 9, 45, 63, 82, 107, and 147. AP activities in *Y. pestis* are given by the numbers beside the circles.

isolated (Fig. 5). *phoA* insertions in the portions of *yscR* encoding the N terminus or the central, hydrophilic domain had low AP activity. Strong AP activity was seen only for the insert after residue 45. On the basis of these data and the properties of the predicted amino acid sequence, we predict that YscR has four membrane-spanning regions and that both the N and C termini as well as a large, centrally located, hydrophilic loop are localized in the cytoplasm (Fig. 5).

Mutant analysis. A previous study had found that polar insertions in the *lcrB* locus that we are now calling *ysc* abolished the growth restriction response (Ca^{2+} requirement for growth) of *Y. pestis* KIM (24). In this study, we characterized a nonpolar, in-frame *yscR* deletion mutant to determine the effect that loss of YscR expression has on the LCR. The growth phenotypes were analyzed for the parent *Y. pestis* KIM5-3001, the YscR$^-$ mutant KIM5-3001.8, and KIM5-3001.8 complemented with *yscR* in *trans* carried on pYPK.R[Q:Δ4-68] (Fig. 6). *Y. pestis* KIM5-3001 displayed a normal Ca^{2+}-dependent phenotype in which the bacteria undergo growth restriction at 37°C in the absence of Ca^{2+}. The mutant *Y. pestis* KIM5-3001.8 showed no growth restriction under these conditions and was therefore characterized as being Ca^{2+} independent in growth. The mutant complemented with *yscR* was restored to the Ca^{2+}-dependent phenotype. (The vector alone had no effect on growth of the parent strain [data not shown].) These data showed that YscR function is necessary for the LCR growth property. A similar finding was made for YscQ, based on the Ca^{2+}-independent growth of the *yscQ* mutant *Y. pestis* KIM5-3001.7 (data not shown).

Expression of V antigen, YopM, and the LCR regulatory protein LcrG from the YscR$^-$ mutant and the complemented strain was studied by immunoblot analysis (Fig. 7). The mutant expressed these proteins at levels similar to those in the parent *Y. pestis* when Ca^{2+} was present but showed no additional induction in the absence of Ca^{2+}. Furthermore, V antigen, YopM, and LcrG proteins were found, as expected, in the supernatant of the parent *Y. pestis* KIM5-3001 cells grown without Ca^{2+} (Fig. 7, lanes b), but they were not secreted by the mutant (lanes d). Both induction in the absence of Ca^{2+} and secretion were restored to parental levels in the complemented strain (lanes f). Hence, the *yscR Y. pestis* was also defective for induction of LCRS operons and secretion of LCRS virulence proteins.

Northern analysis. To determine whether the *yscR* mutation affects LCRS expression at the level of transcription, we compared net transcript abundances for *yopM* and *yscR* itself in the *yscR* strain and the parent *Y. pestis* grown at 37°C (Fig. 8). As in previous studies, we detected smeared RNA bands transcribed from regulatory genes (*yscR*) and defined bands from nonregulatory genes (*yopM*) (5, 21, 44, 55). The transcript levels for both *yopM* and *yscR* were consistent with the protein expression detected by immunoblot analysis. *yopM* message abundance was always greater than that for *yscR*, which appeared to be relatively weakly transcribed. Quantitation of band intensities indicated at least a 27-fold net induction in the absence of Ca^{2+} compared with the presence of Ca^{2+} for YopM message in the parent *Y. pestis*. YopM message in the mutant, however, remained essentially constant irrespective of the presence or absence of Ca^{2+} (Fig. 8, lanes 3 and 4). Similarly, *yscR* message showed little induction in the mutant

FIG. 4. Western blot analysis for the detection of YscR. Lanes: 1 to 4, total membrane preparations; 5 and 6, inner membrane preparations; 7 and 8, outer membrane preparations; 1, 5, and 7, parent *Y. pestis* KIM5-3001 (with Ca^{2+}); 2, 6, and 8, *Y. pestis* KIM5-3001 (without Ca^{2+}); 3, YscR$^-$ mutant *Y. pestis* KIM5-3001.8 (with Ca^{2+}); 4, *Y. pestis* KIM5-3001.8 (without Ca^{2+}).

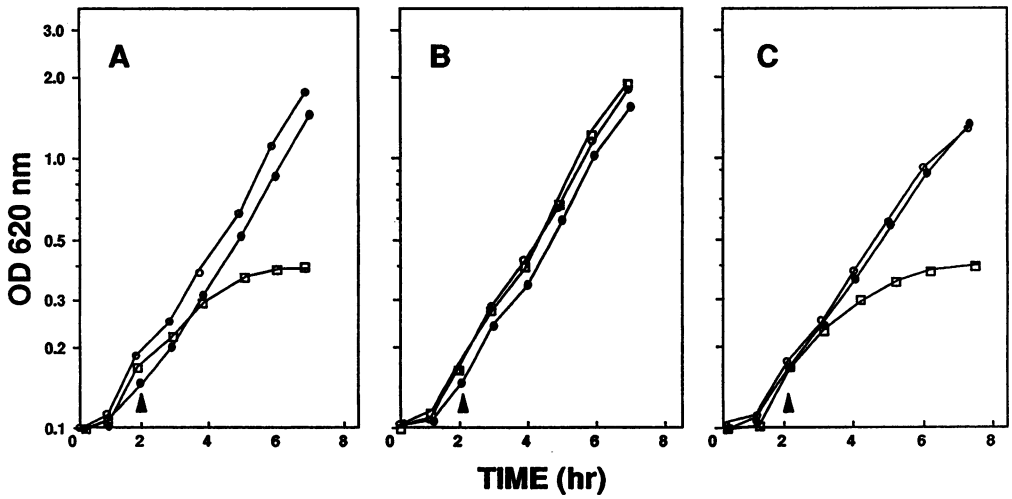

FIG. 6. Growth of the parent *Y. pestis*, the YscR$^-$ mutant, and the mutant complemented with *yscR* in *trans*. Strains were grown in the defined medium TMH, initially at 26°C and later at 37°C (temperature shift indicated by arrowheads). (A) Parent *Y. pestis* KIM5-3001; (B) *yscR Y. pestis* KIM5-3001.8; (C) KIM5-3001.8 complemented with YscR$^+$ pYPK.R (Q:Δ4-68). Open circles, 26°C, no Ca^{2+}; closed circles, 37°C plus 2.5 mM Ca^{2+}; open boxes, 37°C, no Ca^{2+}. OD, optical density.

in the absence of Ca^{2+} (lanes 3 and 4) while being induced at least threefold in *Y. pestis* KIM5-3001 (lanes 1 and 2). These data show that *yscR* itself is subject to Ca^{2+} regulation at the transcriptional level and hence is a member of the LCRS and that this gene is necessary for normal LCR regulation at the level of transcription.

Homologies. All three proteins, YscQ, YscR, and YscS, were found to have homologs in other bacteria (Fig. 9 and Table 2). One group of these was associated with flagellar synthesis. YscQ was homologous to the carboxyl-terminal end of FliY of *Bacillus subtilis* (7), to the switch protein FliN of *E. coli* (40) and *Salmonella typhimurium* (34), and to FliN of *Caulobacter crescentus* (63). YscR and YscS had homology to the flagellum-associated proteins FliP and FliQ, respectively, of *B. subtilis* (8). The second group of homologs contained proteins required for virulence or the presentation of surface antigens in several pathogenic bacteria (26, 29, 70, 81). One group of these, the Mop proteins of *Erwinia carotovora* subsp. *atroseptica*, was necessary both for full virulence on the plant host and for the synthesis of flagella (48).

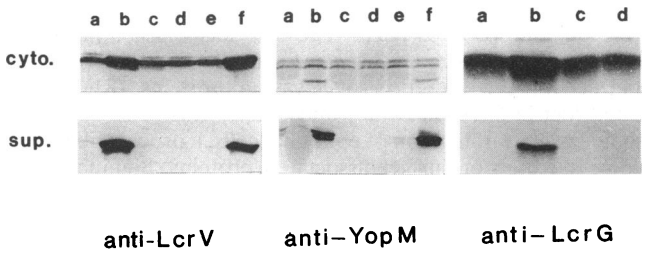

FIG. 7. Western blot analysis of supernatant and cytoplasmic fractions from the parent *Y. pestis*, the YscR$^-$ mutant, and the mutant complemented with *yscR* in *trans*. Lanes: a and b, parent *Y. pestis* KIM5-3001 with and without Ca^{2+}, respectively; c and d, *yscR Y. pestis* KIM5-3001.8 with and without Ca^{2+}, respectively; e and f, fractions from KIM5-3001.8 complemented with YscR$^+$ pYPK.R(Q:Δ4-68) with and without Ca^{2+}, respectively. Antibodies to LcrV, YopM, and LcrG were used to detect the respective proteins in the supernatant (sup.) and soluble (cytoplasm plus periplasm [cyto.]) fractions.

DISCUSSION

The purpose of this study was to further characterize the *lcrB* region of the *Y. pestis* LCR plasmid pCD1. We sequenced a portion of this region and identified three ORFs. We predicted, however, that additional coding sequences existed both up and downstream of *yscQRS*. Our subsequent sequence analysis has confirmed the presence of three ORFs upstream of *yscQRS* (51). Those are being designated *yscN*, *yscO*, and *yscP* so as to follow the lettering sequence ending with *yscM* in the separate *ysc* locus that was previously identified in the original *lcrC* region of pCD1 (*virC* in *Y. enterocolitica*) (24, 27, 44). In addition, our data indicate that at least *yscR* exists in *Y. enterocolitica* and *Y. pseudotuberculosis*, because we isolated and sequenced PCR products homologous to *yscR* from these yersiniae.

Although a preliminary characterization of *yscQ* was made, this study focused on the *yscR* locus. Our regulatory data show that *yscQ* and *yscR* are required for the response to the absence

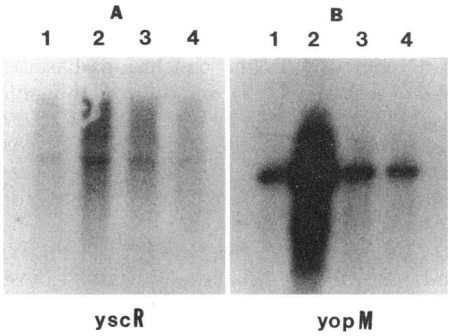

FIG. 8. Northern blot analysis of parent *Y. pestis* KIM5-3001 and the YscR$^-$ mutant *Y. pestis* KIM5-3001.8. Strains were grown at 37°C in TMH in the presence (lanes 1 and 3) and in the absence (lanes 2 and 4) of Ca^{2+}. (A) RNA from *Y. pestis* KIM5-3001 (lanes 1 and 2) and KIM5-3001.8 (lanes 3 and 4) probed with a ^{32}P-labeled PCR product derived from *yscR*; (B) RNA from *Y. pestis* KIM5-3001 (lanes 1 and 2) and KIM5-3001.8 (lanes 3 and 4) probed with a ^{32}P-labeled PCR product derived from *yopM*.

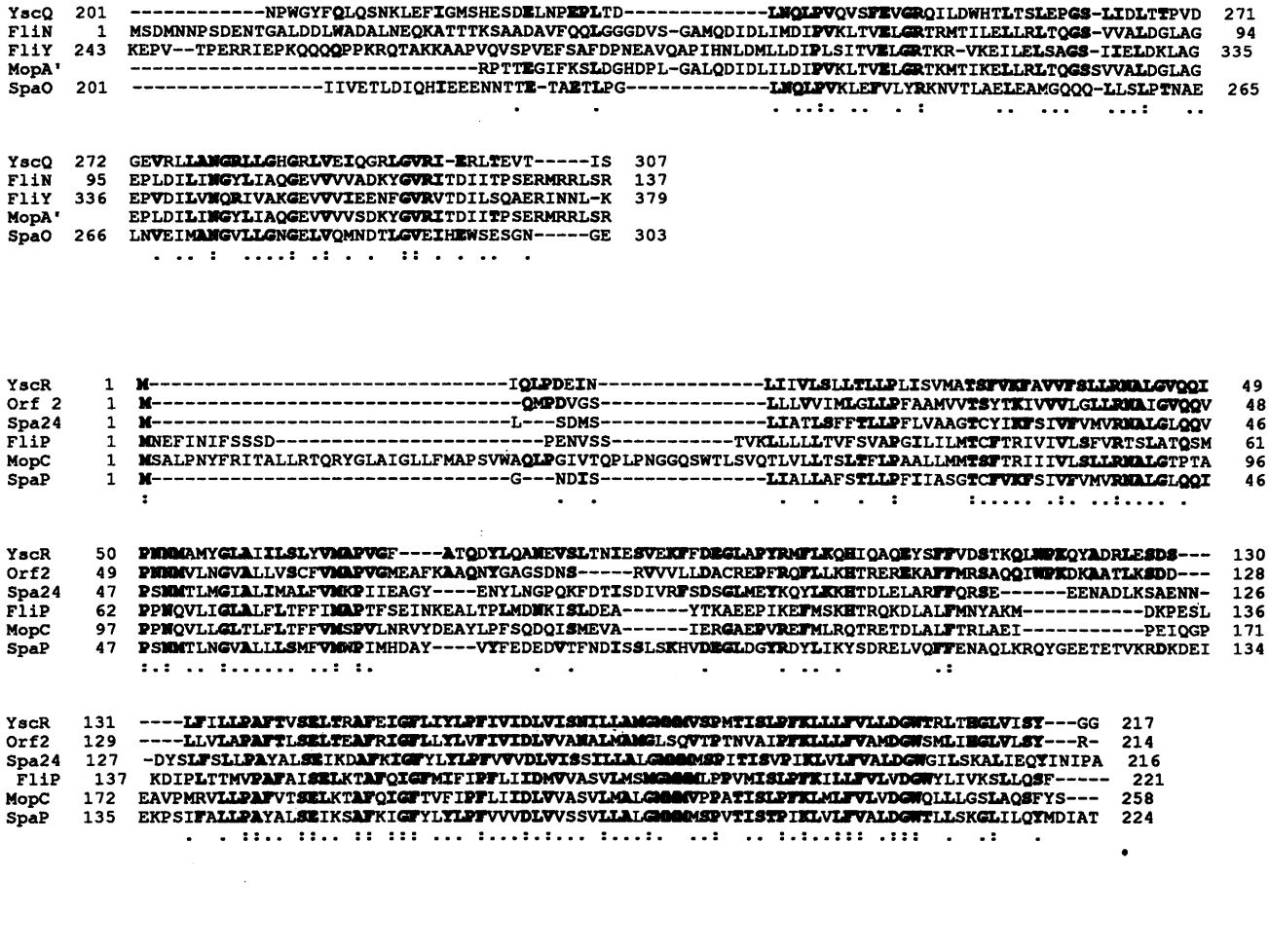

FIG. 9. Alignments of *Y. pestis* genes *yscQ*, *yscR*, and *yscS* with respective homologs. Sequences were aligned by the method of Myers and Miller (50). Dots represent similar residues (amino acid groupings used were AST, DE, NQ, RK, ILMV, FYW); colons indicate that identical residues were present in all of the proteins. Amino acids in boldface are identical to Ysc residues.

of Ca^{2+}, in agreement with the conclusions based on polar MudI1 (Ap^r *lac*) insertions (24). In addition, the *yscR* mutant was unable to secrete normally exported LCRS virulence factors. These data show that *yscR* and probably also *yscQ*, like *lcrD* (54, 55), have dual roles in the LCR, being necessary for full induction and for secretion of V antigen and Yops in the absence of Ca^{2+}.

Like LcrD (2, 22, 30, 46, 62, 68), YscQ, YscR, and YscS have homologs in both virulence-related and flagellar biosynthesis-related systems. YscQ was homologous to proteins that are thought to be part of the switch/motor complex that controls the directional rotation of flagella (7, 31, 32, 34, 40). YscR and YscS were homologous to proteins in *B. subtilis* (8), for which homologs in *S. typhimurium* are required for the formation of the initial rivet portion of the flagellar complex (32).

Virulence-related homologs of the YscQ, YscR, and YscS proteins were found in the *spa* locus of *Shigella flexneri*, in the highly related *spa* locus of *S. typhimurium* (26), and in partially characterized similar loci of enteroinvasive *E. coli* (29) and *E. carotovora* subsp. *atroseptica*. The *spa* locus of *S. flexneri*, located just downstream of *mxiA* (an LcrD homolog), is required for the secretion of invasion proteins (81). The related loci in *S. typhimurium* and enteroinvasive *E. coli* are also necessary for invasion of epithelial cells, a process likely to involve secreted or surface-associated proteins. Significantly, these homologies can, in at least one case, translate into functional complementarity, as the *Shigella spa24*, which is 61% identical to *spaP* in *S. typhimurium*, could complement a noninvasive *S. typhimurium spaP* mutant (26). The homologies extend to virulence systems in nonenteric bacteria, as YscR had 50% identity to ORF2 of the plant pathogen *Xanthomonas campestris*. Although not fully characterized, an ORF2 mutant was nonpathogenic on its plant host (30). Perhaps this protein will also be required for the secretion of virulence factors.

These homologies reinforce the idea that the YscQ, YscR, and YscS proteins may directly function in the secretion of

TABLE 2. Homologs of Ysc proteins[a]

Source of protein	Homolog (% identity)		
	YscQ	YscR	YscS
Virulence related			
S. flexneri	Spa33 (26)	Spa24 (44)	Spa9 (43)
S. typhimurium	SpaO (31)	SpaP (46)	SpaQ (44)
E. coli (entero-invasive)			InvX (43)
E. carotovora[b]	MopA (20)	MopC (37)	MopD (33)
X. campestris		ORF2 (50)	
Flagellum related			
B. subtilis	FliY (30)	FliP (33)	FliQ (36)
E. coli	FliN (31)		
S. typhimurium	FliN (31)		
C. crescentus	FliN (22)		

[a] YscQ is homologous only to the carboxyl termini of Spa33, SpaO, and FliY, and the alignments were optimized by using only the carboxyl termini (see Fig. 9). The reported MopA sequence includes only a portion of the carboxyl terminus, and that was used in the alignment with the carboxyl terminus of YscQ (see Fig. 9).

[b] The Mop proteins in *E. carotovora* subsp. *atroseptica* are necessary for both virulence and expression of flagella (48).

LCRS virulence proteins. We predict that there also will be additional components to the Yop transport system, such as an analog of FliI (Spa47 in *S. flexneri* [81] or SpaL in *S. typhimurium* [26]) that may function to energize transport (32, 81), and indeed one of the ORFs upstream of *yscQ* (*yscN*) is homologous to *fliI* (51). Moreover, our partial sequencing downstream of *yscS* has revealed homology to *spa29*, which is the *spa* gene downstream of *spa9* in *S. flexneri* (corresponding to *mopE* in *E. carotovora* subsp. *atroseptica* [48]) (51). We hypothesize that YscQRS (LcrD) may form a complex, given the precedent of a basal body-associated complex that evidently contains some of the homologs of *lcrB*-encoded Ysc proteins (31) and on findings indicating that LcrD interacts with another protein in its function (55). Because of the inner membrane location of YscR and LcrD and the predicted multiple membrane-spanning regions in both proteins, our working model involves these in transport of V antigen and Yops through the inner membrane. It is striking that the sequences in the vicinity of the putative membrane-spanning regions are highly conserved among the YscR homologs (Fig. 9) as is the case for the LcrD homologs (54). This suggests conservation of function beyond that of stable insertion in the membrane; such regions could also be involved in the formation of the hypothetical translocase complex or in the transport process itself. The less conserved putative cytoplasmic loop region in YscR and its homologs could have diverged in sequence to serve system-specific functions.

Some of the proteins encoded by the previously characterized *ysc* operon in the *lcrC* region of pCD1, particularly the YscC homolog of PulD (44, 61), may function in transport through the outer membrane (by analogy to PulD function), but others with putative transmembrane domains, such as YscD or YscG, may prove to have an inner membrane location. At least some Ysc homologs are also present in other bacterial systems (1, 25, 61). It may be that many bacterial systems have a secretion mechanism composed of YscQRS (LcrD)-like and other Ysc-like elements.

It is not known why this secretion system is so complex. Some of the complexity may come from incorporating a mechanism for regulating the synthesis of the secretion system itself as well as for regulating synthesis of the secretion substrates. Such a concept has a strong precedent in the flagellar biogenesis system, in which the synthesis, secretion, and assembly of sequential components of the organelle are closely orchestrated (32). In addition, the LCR-associated secretion system is complex, because it interfaces with, or even incorporates mechanisms for sensing and responding to, environmental signals.

In the LCR, LCRS operons are thermally induced (15, 16, 78, 82), and maximal expression occurs in the absence of Ca^{2+} or nucleotides (78). Ca^{2+} or nucleotides cause a partial downregulation of LCRS operon expression, and Ca^{2+} has been shown also to impose a strong block on release of LCRS-encoded virulence proteins from the bacteria. *yscR*, *lcrD*, and *yscBCDEF* themselves belong to the LCRS (27, 54) along with the operons encoding V antigen and Yops, and all of these secretion-associated genes in turn are necessary for the normal LCR regulation at the transcriptional level (24, 27, 44, 54, 55). Mutations abolishing the function of LcrD and YscR and probably also YscQ cause partial downregulation (repression) in the absence as well as the presence of Ca^{2+}. This finding suggests that the downregulating pathway is potentially functional in the absence of Ca^{2+} but that YscQRS (LcrD) normally may act to prevent its negative effects (78).

Accordingly, we speculate that YscQRS (LrD) is multifunctional, directly participating in regulation of LCRS operon expression as well as in secretion of V antigen and Yops. This hypothesis is consistent with our inability to separate these roles for LcrD by genetic means (55, 56). Even single base changes distributed throughout LcrD affect both functions (56). Even so, LcrD is essential for secretion of Yops independently of its role in transcriptional regulation, because YopM expressed from the *trc* promoter is not secreted by an LcrD$^-$ *Y. pestis* mutant (56).

This study has added to our picture of the secretion-regulatory mechanism that may operate in diverse pathogenic systems by identifying new components of this mechanism and characterizing regulatory effects of YscR.

ACKNOWLEDGMENTS

This study was supported by PHS grant AI21017. G. V. Plano was supported by NRSA F32 AI08525.

Synthetic oligonucleotides and peptides were made by Mike Russ of the University of Kentucky Macromolecular Structure Analysis Facility.

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